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WO 9603144A1

(51) International Patent Classification 6 :

A61K 39/00, 39/395, C12N 15/86, 15/12,
15/13, C07K 19/00

(43) International Publication Date: 8 February 1996 (08.02.96)

(21) International Application Number: PCT/AU95/00461

(22) International Filing Date: 27 July 1995 (27.07.95)

(30) Priority Data:

PM 7079 27 July 1994 (27.07.94) AU
PN 1009 8 February 1995 (08.02.95) AU

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(54) Title: POLYPEPTIDE VACCINES

(57) Abstract

The present invention relates to a recombinant polyepitope cytotoxic T lymphocyte vaccine. The vaccine comprises at least one recombinant protein including a plurality of cytotoxic T lymphocyte epitopes from one or more pathogens, wherein the at least one recombinant protein is substantially free of sequences naturally found to flank the cytotoxic T lymphocyte epitopes. In addition the present invention also provides a polynucleotide including at least one sequence encoding a plurality of cytotoxic T lymphocyte epitopes from one or more pathogens.

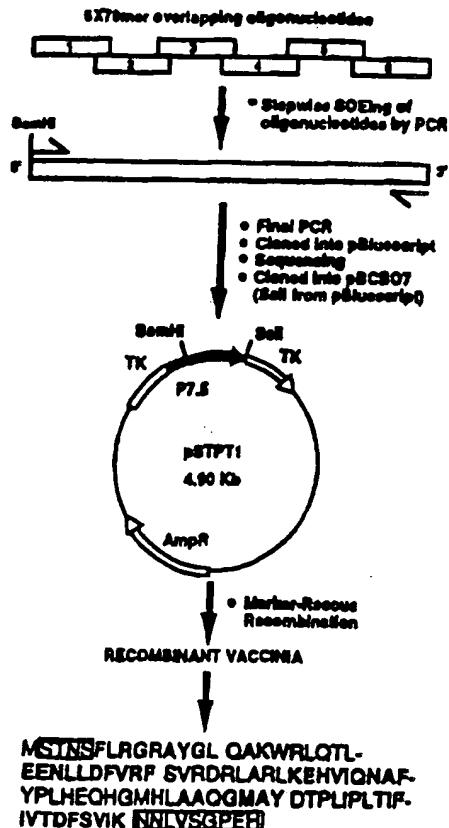
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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

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With international search report.



Polytope Protein Sequence

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POLYPEPTIDE VACCINES

The present invention relates to vaccines containing a plurality of cytotoxic T lymphocyte epitopes and to polynucleotides including sequences encoding a plurality 5 of cytotoxic T lymphocyte epitopes.

CD8 + $\alpha\beta$ cytotoxic T lymphocytes (CTL) recognise short peptides (epitopes, usually 8-10 amino acids long) associated with specific alleles of the class I major histocompatibility complex¹ (MHC). The peptide epitopes 10 are mainly generated from cytosolic proteins by proteolysis, a process believed to involve the multicatalytic proteosome complex²⁻⁷. Peptides of appropriate length are transported into the endoplasmic reticulum where specific epitopes associate with MHC. The 15 MHC/epitope complex is then transported to the cell surface for recognition by CTL. The influence of sequences flanking CTL epitopes on the proteolytic processing of these epitopes remains controversial⁸⁻¹². However, by constructing a recombinant vaccinia coding for 20 an artificial polypeptide protein containing nine CD8 CTL epitopes in sequence, the present inventors have determined that the natural flanking sequences of CTL epitopes are not required for class I processing, that is each epitope within the polyepitope protein was always 25 efficiently processed and presented to appropriate CTL clones by autologous polyepitope vaccinia infected target cells.

Accordingly, in a first aspect, the present invention consists in a recombinant polyepitope cytotoxic 30 T lymphocyte vaccine, the vaccine comprising at least one recombinant protein including a plurality of cytotoxic T lymphocyte epitopes from one or more pathogens, wherein the at least one recombinant protein is substantially free of sequences naturally found to flank the cytotoxic T 35 lymphocyte epitopes.

Preferably, the at least one recombinant protein does not include any sequences naturally found to flank the cytotoxic T lymphocyte epitopes. However, it should be understood that small lengths (e.g. 1-5 amino acids) of sequences naturally found to flank the cytotoxic T lymphocyte epitopes may be included. The phrase "substantially free of sequences naturally found to flank the cytotoxic T lymphocyte epitopes" is to be taken as including such short lengths of flanking sequences.

In a second aspect, the present invention consists in a polynucleotide, the polynucleotide including at least one sequence encoding a plurality of cytotoxic T lymphocyte epitopes from one or more pathogens, and wherein the at least one sequence is substantially free of sequences encoding peptide sequences naturally found to flank the cytotoxic T lymphocyte epitopes.

Again, it is to be understood that "substantially free of sequences encoding peptide sequences naturally found to flank the cytotoxic T lymphocyte epitopes" includes the possibility of including short peptide (e.g 1-5 amino acids) sequences naturally found to flank the cytotoxic T lymphocyte epitopes.

In a third aspect, the present invention consists in a nucleic acid vaccine, the vaccine comprising the polynucleotide of the second aspect of the present invention and an acceptable carrier.

In a fourth aspect, the present invention consists in a vaccine formulation, the vaccine comprising the recombinant protein of the first aspect of the present invention and an acceptable carrier and/or adjuvant.

In a preferred embodiment of the present invention the at least one recombinant protein includes, and the at least one sequence encodes, at least three cytotoxic T lymphocyte epitopes.

In a further preferred embodiment, the epitopes are from multiple pathogens.

It is also envisaged that the vaccines according to the invention may include immunomodulatory compounds (such as cytokines), other proteins/compounds (such as melittin or regulatory proteins) and/or adjuvants. The vaccines 5 may also include helper epitopes/CD4 epitopes and proteins, B-cell epitopes or proteins containing such epitopes, for example tetanus toxoid. Another example of a vaccine according to the invention comprises a recombinant vaccine construct wherein the polytope 10 including the CTL epitopes is linked to an extracellular glycoprotein or glycoproteins containing B-cell and/or CD4 epitopes.

The vaccines according to the invention may be delivered by various vectors, for example vaccinia 15 vectors, avipox virus vectors, bacterial vectors, virus-like particles (VLP's) and rhabdovirus vectors or by nucleic acid vaccination technology. As polytope proteins are likely to be sensitive to proteolysis during manufacture and/or serum following injection, we envisage 20 that such vaccines may best be delivered using nucleic acid vaccination technologies¹², vector systems or adjuvant systems which protect the polytope protein from proteolysis. Additional information regarding vectors may be found in Chatfield *et al*, Vaccine 7, 495-498, 1989; 25 Taylor *et al*, Vaccine 13, 539-549, 1995; Hodgson "Bacterial Vaccine Vectors" in *Vaccines in Agriculture*.

A polytope vaccine according to the invention may also include a large number of epitopes (e.g. up to 10 or more) from one pathogen so that the HLA diversity of the 30 target population is covered. For instance a vaccine containing epitopes restricted by HLA A1, A2, A3, A11 and A24 would cover about 90% of the Caucasian population.

A polytope vaccine according to the invention may also be constructed such that the multiple epitopes are 35 restricted by a single HLA allele.

In a preferred embodiment of the fourth aspect of the present invention the vaccine formulation includes ISCOMs. Information regarding ISCOMs can be found in Australian patent No 558258, EP 019942, US4578269 and 5 US4744983, the disclosures of which are incorporated herein by reference.

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following 10 examples and accompanying Figures in which:-

Figure 1. Construction of a recombinant vaccinia that expresses a synthetic DNA insert coding for the polytope protein, which contains nine CTL epitopes in sequence. Boxed sequences representing linear B cell 15 epitopes.

Figure 2. CTL recognition of each epitope expressed in the recombinant polytope vaccinia construct.

Figure 3. Polytope vaccinia can recall epitope specific responses. Bulk effectors from donors 20 (A) CM - A24, A11, B7, B44; (B) YW - A2, B8, B38 and (C) NB - A2, A24, B7, B35 were generated by infecting peripheral blood mononuclear cells (PBMC) with the polytope vaccinia at a MOI of 0.01 for 2 hours followed by 25 2 washes. After 10 days culture in 10% FCS/1640 RPMI the bulk effectors were used against autologous phytohaemagglutinin T cell blasts target cells (E:T 20:1) sensitised with the indicated peptide (10 μ M) in a standard 30 5 hour chromium release assay¹⁴. Bulk effectors generated by the addition of irradiated autologous A type LCLs¹⁴ (LCL to PBMC ratio 1:50) gave similar results to that shown above.

Figure 4 shows the construction of a polytope DNA insert including ten murine CTL epitopes.

Figure 5 shows the sequence of the polytope DNA 35 insert of Figure 1.

Figure 6 provides results of CTL assays conducted on splenocytes harvested from mice vaccinated with recombinant vaccinia including the DNA insert of Figure 3.

Figure 7 shows comparison of spleen MCMV titres
5 (± standard error) 5 weeks after polytope vaccinia vaccination and 4 days after MCMV challenge. P values - unpaired student t-test

Figure 8 DNA vaccination with different plasmids in Balb/c mice.

10 Figure 9. Murine Polytope vaccinia immunised (IP) mice from these strains (Balb/c, CBA, C56BL/6) had the spleens removed and splenocytes restimulated with peptide (eg for A and A'). effectors were generated by stimulation with influenza NP peptide "TYQRTRALV"). The effectors
15 were then used on peptide coated targets (A-J), virus infected targets (A'-J') and tumour targets (I'). Virus infected targets were either infected (A', F'), with allantoic fluid as negative control or murine polytope vaccinia (Vacc Mu PT) (B'-D', F'-J'), with human polytope
20 vaccinia (Vacc Hu PT) as the negative control.

Example 1

Nine, class I restricted, CTL epitopes from several Epstein-Barr virus nuclear antigens (EBNA) have previously
25 been defined using CTL clones^{10, 18-20}. The clones were isolated from a panel of normal healthy Epstein-Barr virus (EBV) seropositive donors and were restricted by different HLA alleles (Table 1). A recombinant polyepitope vaccinia (polytope vaccinia), which coded for a single artificial
30 protein containing all nine of these CTL epitopes, was constructed (see Fig. 1). The DNA sequence coding for this protein was made using splicing by overlap extension (SOEing) and the polymerase chain reaction (PCR) to join six overlapping oligonucleotides. The insert was cloned
35 into pBluescript II, checked by sequencing and transferred into pBCBO7¹⁵ behind a vaccinia promoter to generate

pSTPT1. This plasmid was then used to generate the polytope vaccinia virus by marker-rescue recombination¹⁶. The artificial polytope protein expressed by this vaccinia therefore containing no sequences naturally found to flank 5 the CTL epitopes in their proteins of origin (Fig. 1).

CTL CLONES	COGNATE EPITOPES	SOURCE	HLA	REFS
			RESTRICTION	
LC13	FLRGRAYGL	EBNA3	B8	13
LC15	QAKWRLQTL	EBNA3	B8	14
CS31	EENLLDFVRF	EBNA6	B44	15
YW22	SVRDRRLARL	EBNA3	A0203	14
CM4	KEHVIQNAF	EBNA6	B44	13
NB26	YPLHEQHGM	EBNA3	B3501	14
LX1*	HLAAQGMAY	EBNA3	?	14
JSA2	DTPLIPLTIF	EBNA3	B51 ^f /A2	13
CM9	IVTDFSVIK	EBNA4	A11	16

Table 1: Description of the CTL clones, their cognate epitopes, the proteins of origin (source) and their HLA restriction. The first two letters of the clones refer to the donors. *Not tested (see Fig. 2). ^fRecent evidence suggests this epitope may be restricted by HLA-B51. All the epitopes have been minimalised except EENLLDFVRF and DTPLIPLTIF.

10

A DNA sequence coding for the polytope amino acid sequence was designed with codons most frequently used in mammals and incorporated a Kozac sequence¹³ and a BamHI site upstream of the start codon. Six 70mer

15 oligonucleotides overlapping by 20 base pairs were spliced together using Splicing by Overlap Extension (SOEing)¹⁴ in 20μl reactions containing standard PCR buffer, 2mM MgCl₂, 0.2mM dNTPs, 1.5U of Taq polymerase (hot start at 94°C) using the following thermal program: \94°C for
20 10 seconds, 45°C for 20 seconds and 72°C for 20 seconds (40 cycles). Half of each gel purified dimer sample was combined in a second PCR reaction (12 cycles) with the addition of 0.5μl of α³²p dCTP. The reaction was run on a 6% acrylamide gel and a slice corresponding to the
25 position of the hexamer product was isolated. Two 20mer

oligonucleotides were used to PCR amplify the hexamer using an annealing temperature of 56°C and 25 cycles. The gel purified fragment was cloned into the EcoRV site of pBluescript II KS-, was checked by sequencing and cloned 5 behind the vaccinia P7.5 early/late promoter using the BamHI/SalI sites in the vaccinia plasmid vector pBCB07¹⁵ to generate pSTPT1. Construction of a TK- recombinant virus was carried out using marker rescue combination between pSTPT1 and VV-WR-L929 as described previously¹⁶.
10 Plaque purified virus was tested for TK phenotype and for appropriate genome arrangement by Southern blotting of viral DNA¹⁶.

To establish whether each epitope could be efficiently processed from the polytope protein the 15 polytope vaccinia was used to infect a panel of target cells, which expressed the HLA alleles restricting each epitope. Autologous CTL clones specific for each epitope were then used as effector cells in standard chromium release assays. In all cases tested the CTL clones 20 recognised and killed the HLA matched target cell infected with the polytope vaccinia and the appropriate (see Table 1) EBNA vaccinia (positive controls), but not the TK-vaccinia (negative controls) (Fig. 2).

Figure 2 shows CTL recognition of each epitope 25 expressed in the polytope vaccinia construct. Effector CTL are listed in Table 1 (E:T ratio 5:1). Target cells (see below) were infected with recombinant vaccinia expressing either (i) the EPV nuclear antigen (EBNA) recognised by the CTL clone (see Table 1) (positive 30 control), (ii) TK- (negative control), or (iii) the polytope construct (i.e., Polytope vaccinia). Vaccinia infection of the target cells was at a multiplicity of infection of 5:1 followed by 14-16 hour incubation at 37°C prior to use in the standard, 5 hour, ⁵¹Cr-release 35 assay²⁹. Clone LX1 was no longer available at the time of assay. Target cells; there are two types of EBV, A and B-

type, whose EBNA protein sequences differ significantly. CTL clones LC13, LC15, CM4, NB26, JSA2 and CM9 recognise cells transformed with A-type EBV but not B-type EBV, and CTL clones CS31 and YW22 recognise cells transformed with 5 A-type EBV and EBV^{10, 18-20}. The target cells used for the A-type specific CTL were therefore autologous lymphoblastoid cell lines transformed with B-type virus (B-type LCLs). The target cell for CS31 and YW22 were EBV negative B cell blasts, established using anti-CD40 10 antibody and rIL-4²¹.

An additional series of experiments used the polytope vaccinia to stimulate *in vitro* a secondary CTL response from peripheral blood mononuclear cells (PBMC) obtained from healthy EBV seropositive donors. The 15 resulting bulk CTL cultures were then used as effectors against peptide epitope sensitised autologous PHA blasts in a standard chromium release assay. The polytope vaccinia was capable of recalling CTL responses which were specific for epitopes restricted by the HLA alleles 20 expressed by each donor (Fig. 3).

Figure 3 shows that polytope vaccinia can recall epitope specific responses. Bulk effectors from donors (A) CM - A24, A11, B7, B44; (B) YW - A2, B8, B38 and (C) NB - A2, A24, B7, B35 were generated by infecting 25 peripheral blood mononuclear cells (PBMC) with the polytope vaccinia at a MOI of 0.01 for 2 hours followed by 2 washes. After 10 days culture in 10% FCS/1640 RPMI the bulk effectors were used against autologous phytohaemagglutinin T cell blasts target cells (E:T 20:1) 30 sensitised with the indicated peptide (10μM) in a standard 5 hour chromium release assay¹⁹. Bulk effectors generated by the addition of irradiated autologous A type LCLs¹⁹ (LCL to PBMC ratio 1:50) gave similar results to that shown above.

35 Two linear B cell epitopes (STNS and NNLVSGPEH) recognised by monoclonal antibodies (8G10/48²² and

8E7/55²³ respectively) were incorporated at each end of the polytope construct (Fig. 1) to follow the expression of the polytope protein. Western blotting and indirect immunofluorescence antibody staining of polytope vaccinia 5 infected lymphoblastoid cell lines (LCLs) and the processing defective T2 cell line^{6,7} using these antibodies failed to detect polytope protein products (data not shown). Recombinant proteins expressed by vaccinia using the same P7.5 promoter are usually readily 10 detected²⁴ implying that the polytope protein was rapidly degraded in the cytoplasm of mammalian cells. This degradation was not dependent on LMP2 and 7 since the T2 cell line does not express these proteosome associated 15 endopeptidases^{6,7}. This phenomenon is consistent with other studies expressing truncated proteins or peptides in mammalian cells²⁵ and is likely to reflect the inability of such proteins to fold into any secondary or tertiary 20 structures.

A glutathione S-transferase fusion expression vector containing the human polytope was constructed. The DNA 25 fragment coding for the human polytope was excised from pBSpolytope using BamHI/HincII and cloned into the BamHI/AmaI restriction sites in pGex-3x (GST Gene Fusion System Pharmacia) to make pFuspoly. This plasmid was used to express the polytope fusion in bacteria using the standard induction protocols. An aliquot of the bacteria 30 was lysed in loading buffer and run on a 20% SDS PAGE gel with size markers. This gel indicated that the expected protein of approximately 38kD (the human polytope plus the GST domain (26kD)) was being expressed in bacteria containing the plasmid. Western blotting with the two 35 monoclonal antibodies 8G10/48 and 8E7/55 demonstrated that the fusion detected contained the human polytope which has the two linear B cell epitopes (STNS and NNLVSGPEH respectively) incorporated at each end of the polytope

construct. This protein may be incorporated into liposomes or ISCOMs.

Attempts to purify the fusion protein using the GST purification employing glutathione agarose beads failed due to the lack of fusion protein in the bacterial extract supernatant. All the fusion protein precipitated with the cell debris. The protocol was not at fault since GST expressed by itself in a different bacterial culture was in the bacterial extract supernatant and could be purified easily. These data suggest the fusion protein is rapidly degraded in the bacteria unless sequestered into bacterial inclusion bodies from which purification using the GST system is difficult.

15 *Example 2*

MATERIALS AND METHODS

20 **Construction of a recombinant vaccinia expressing the murine polytope protein.** Ten class I murine CTL epitopes from various diseases were selected so that there were two epitopes for each of H-2Db, H-2Kb, H-2Kd, H-2Kk and H-2Ld which are represented in three strains of mice (see Table 2). These amino acid sequences were arranged 25 such that each of the first 5 epitopes was restricted by a different HLA allele followed by the second group 6-10. The two groups of epitopes were converted to a DNA sequence using the universal codon usage data. These two DNA sequences were separated by an SpeI and flanked by a 30 XbaI restriction site at the 5' end and a AvrII site at the 3' end. Also incorporated at the 5' end is a BamHI restriction site, a Kozac sequence¹³ and a methionine start codon. While at the 3' end there is a B cell epitope from *Plasmodium falciparum*, a stop codon and a 35 Sall restriction site see Figures 4 and 5. Five 75mer oligonucleotides and a 76mer oligonucleotide overlapping

by 20 base pairs, representing this 341 base pair sequence, were spliced together using Splicing by Overlap Extension (SOEing)¹⁴ and the polymerase chain reaction (PCR). Primer dimers were made of primers 1 and 2, 3 and 4, 5 and 6 (0.4µg of each) in 40µl reactions containing standard 1x *Pfu* PCR buffer, 0.2 mM dNTPs and 1U of Cloned *Pfu* DNA polymerase (hot start at 94°C) using a Perkin Elmer 9600 PCR machine programmed with the following thermal program; 94°C for 10 seconds, 42°C for 20 seconds and 72°C for 20 seconds for 5 cycles. At the end of 5 cycles the PCR programme was paused at 72°C and 20µl aliquots of reactions 2 and 3 were mixed (reaction 1 was left in the PCR machine) and subjected to a further 5 cycles. At cycle 10 the program was paused again and 20µl of reaction 1 added to the combined reactions 2 and 3 and a further 5 cycles completed. The combined 40µl sample was then gel purified on a 4% Nusieve agarose gel (FMC) and a gel slice corresponding to the correct sized fragment removed and spun through Whatmann 3MM paper. Two 20mer oligonucleotides were used to PCR amplify the full length product using the standard reaction mix as above and an annealing temperature of 50°C and 25 cycles. The full length PCR fragment was gel purified in a 4% Nusieve agarose gel, cloned into the EcoRV site of pBluescript IIKS⁻ to make pBSMP and checked for mutations by sequencing. The DNA insert of a plasmid containing the correct sequence was excised using BamHI/SalI restriction enzymes and cloned, using the same enzymes, behind the vaccinia P7.5 early/late promoter in the plasmid shuttle vector pBCB07¹⁵ to generate pSTMOUSEPOLY. Construction of a TK- recombinant virus was carried out using marker rescue recombination between pSTMOUSEPOLY and VV-WR-L929 using protocols described previously¹⁶. Plaque purified virus was tested for TK phenotype and for appropriate genome arrangement by Southern blotting of viral DNA¹⁷.

Vaccination of mice with recombinant murine polytope vaccinia. The recombinant vaccinia was used to vaccinate 3 mice in each of the 3 strains of mice Balb/cv, C57BL/6 and CBA. The vaccinations were I.V. 50 μ l containing 5×10^7 pfu of vaccinia and the mice were left to recover for three weeks. The TK- vaccinia was used as a negative control for each strain of mouse in this experiment.

10 Cytotoxic T cell assays. Splenocytes were harvested from the vaccinated mice 3 weeks post vaccination and restimulated with the appropriate peptides (1 μ g/ml) in vitro¹⁶. No peptide were used for restimulations as negative controls. After 7 days of culture the 15 restimulated bulk effectors were harvested and used in a 5 hour, ⁵¹Cr-release assays. The targets used in these assays were ConA blasts generated from each of the strains coated with one of the peptides presented by that strain. Three effector to target ratios were used 50:1, 10:1 and 20 2:1 the results are shown in Figure 6.

RESULTS

Construction of murine recombinant polytope vaccinia, 25 The list of epitopes included in the murine polytope are listed in Table 2.

Table 2 CTL epitopes of the murine CTL polytope

SOURCE	SEQUENCE	RESTRICTION	MOUSE STRAIN
Influenza nuclear protein (366-374)	ASNENMDAM	H-2D ^b	C57BL/6
Adenovirus 5 E1A (234-243)	SGPSNTPPEI	H-2D ^b	C57BL/6
Ovalbumin (257-264)	SIINFEKEL	H-2K ^b	C57BL/6
Sendai virus nuclear protein (324-332)	FAPGNYPAL	H-2K ^b	C57BL/6
Influenza nuclear protein (147-155)	TYQRTRALV	H-2K ^d	Balb/c
P. Berghei Circumsporozoite protein (249-257)	SYIPSAEKI	H-SK ^d	Balb/c
Influenza nuclear protein (50-58)	SDYEGRILL	H-2K ^k	CBA
Influenza NS1 (152-160)	EEGAIIVGEI	H-SK ^k	CBA
Murine Cytomegalovirus pp89 (168-176)	YPHFMPTNL	H-2L ^d	Balb/c
Lymphocytic choriomeningitis virus nuclear protein (118-126)	RPQASGVYM	H-SL ^d	Balb/c

The construction of the polytope DNA insert is summarised
5 in Fig. 4. The polytope sequence is shown in Fig 5.

CTL assays.

Each epitope in the polytope induced a primary CTL response in mice with the appropriately MHC allele. No
10 competition between two epitopes restricted by the same allele was observed. (the high flu NP response in CBA mice given TK- controls is likely to be due to a naturally acquired influenza).

15 Polytope constructs containing multiple CTL epitopes from various pathogens restricted by various MHC alleles

are clearly capable of generating primary CTL responses to each epitope within the polytope vaccine. This has clear application in all vaccines where CTL responses are required for protection. For instance, multiple HIV CTL epitopes might be combined in a therapeutic vaccine to foreshadow epitopes expressed by escape mutants and thereby prevent disease progression.

5 Murine polyepitope mice have SIINFEKL specific CTL which can kill the ovalbumin transfected cell line EG7 in vitro and in vivo.

SIINFEKL specific CTL which kill the EG7 tumor cells demonstrated in vitro

15 Spleen cells from murine vaccinia immunised mice were collected 4 weeks post vaccination and restimulated in vitro with 10ug/ml SIINFEKL for 7 days. Effectors could not lyse the untransfected parent line EL4 but could lyse the EG7 tumour cells and EL4 cells sensitised with 20 SIINFEKL.

Protection against EG7 tumour in vivo afforded by murine polytope

25 Mice (C57B6) were given either human polytope vaccinia (Thomsom et al., 1995) or murine polytope vaccinia (10^7 pfu/mouse/ip) and 4 weeks later received 10^7 EL4 or EG7 tumour cells (Moore et al., 1988. Cell 54, 777) subcutaneously (10 or 11 mice per group).

30 The number of mice with visable tumours (all were $>1\text{cm}$ diameter) at day 9 is given.

Human Polytope Vaccinia		Murine Polytope Vaccinia	
EG7	EL4	EG7	EL4
10/10*	10/10	0/11	10/10

* (Two mice had tumours $<1\text{cm}$ in diameter)

Protection against MCMV

BALB/c mice were challenged with MCMV (K181 strain, 8 x 10³ PFU, 100µl intraperitoneally) 5 weeks after 5 polytope vaccinia vaccination. Four days after challenge the viral titres per gram of spleen were determined the results are shown in Fig. 7 (method of Scalzo et al)¹⁷.

10 *Evaluation of polytope vaccines delivered in a DNA plasmid.*

The polytope protein described above contained a linear antibody epitope recognised by a monoclonal antibody. As described above the polytope protein could not, however, be detected in cells infected with the 15 polytope vaccinia indicating that it is very unstable; a likely consequence of having no folding structure. It was thus considered that delivery of a polytope vaccine may be best achieved using nucleic acid vaccination technology or with an adjuvant system that protects from proteolysis 20 (eg liposomes or ISCOMs).

The CMV promotor cassette from pCIS2.CXXNH (Eaton et al (1986) *Biochemistry* 25(26) p8343) was cloned into the EcoRI site of pUC8 in the same orientation as the LacZ gene to make the plasmid pDNAVac (used as a control 25 plasmid in the DNA vaccination experiments). This plasmid then had the murine polytope (from pBSMP) inserted into the XhoI site in the multiple cloning site to form pSTMPDV. The plasmid pRSVGM/CMVMP has fragments sourced from a number of different plasmids. The RSV promotor was 30 excised from pRSVHygro (Long et al (1991) *Hum. Immunol.* 31, 229-235), the murine GM-CSF gene from pMPZen(GM-CSF) (Johnson et al (1989) *EMBO* 8, 441-448) and the CMV promotor cassette from pCS (Kienzie et al (1992) *Arch. Virol.* 124 p123-132). Into the CMV cassette was the 35 murine polytope cloned into the SmaI site of the multiple

cloning site. Both genes , murine GM-CSF and the murine polytope, use the bi-directional polyA from SV40.

Nine 6 week old female Balb/c mice were injected I.M. with 50 μ g of either pDNAVac (plasmid control), 5 pSTMPDV (murine polytope only) or pRSVGM/CMVMP (murine GM-CSF and murine polytope) in 50 μ l of PBS (see next figure). They were given boosters with another 50 μ g of the same plasmids at 3 weeks. At 8 weeks from the vaccination these mice were killed and their spleens removed.

10 Splenocytes were isolated and cultured with peptide as previously described for vaccinia vaccinated animals. These bulk effectors were then used in standard ^{51}Cr release assays against P815 cells coated with peptide corresponding to the epitopes in the murine polytope that

15 are presented by Balb/c mice. The assay was done for 6 hours at E:T ratios of 2:1, 10:1 and 50:1.

The results of these experiments are shown in Fig 8.

**SPECIFIC CTL ACTIVITY AGAINST PEPTIDE COATED AND VIRUS
20 INFECTED TARGETS INDUCED BY THE MURINE POLYTOPE VACCINIA**

Method

1. Vaccination and Effector Cell Preparation. Mice (3 per group) were vaccinated intraperitoneally (IP) with 5 x 25 10^7 PFU vaccinia. Mice were boosted via the same route and with the same amount of vaccinia week 3. The spleens were removed 6 weeks after the initial vaccination and the splenocytes were isolated after erythrocyte lysis with ACK Buffer (0.15M NH₄Cl, 1mM KHCO₃, 0.1mM Na₂EDTA) (Current 30 Protocols in Immunology, Ed JE Coligan, AM Kruisbeek, DH Margulies, EM Shevach, W Strober, 1994 John Wiley and Sons Inc. USA.). 5 x 10^6 splenocytes per well were peptide restimulated (1 μ g/ml) in bulk T cell media (RPMI/10% Fetal Calf Serum (FCS), 2mM Glutamine, 5x10⁻⁵M 35 2-Mercaptoethanol) for seven days prior to cytotoxic T

lymphocyte (CTL) assay on $^{51}\text{chromium}$ (^{51}Cr) labelled target cells¹⁷. The peptides used for restimulation are given above A to J. The effectors were used against either peptide coated targets A-J, viral infected targets 5 (A'-J') or transfected antigen expressing targets (I').

2. Preparation of Target Cells. Cell lines used as targets in these assays were P815 for Balb/c (H-2^d), EL-4 and EG7 for C57BL/6 (H-2^b), L929 for CBA (H-2^k) L929, or con A blasts prepared from the Balb/c, C57BL/6 or CBA 10 mice, respectively.¹ To express the required epitope for CTL killing, target cells were either pre-incubated with (i) peptide (A-J), (ii) vaccinia (B'-D', F'-J'). or (iii) Influenza (A', E'), or maintained as the (iv) Ovalbumin-expressing plasmid transfectant of El-4 (EG7) in the case 15 of the SIINFEKL epitope system (I').

(i) Peptide coated targets (A-J): Target cells were centrifuged at 1000rpm/5 min. The supernatant was discarded to approximately 200 $\mu\text{g}/\text{ml}$ and 10-20 μl of either 20 RPMI (No peptide) or 200 $\mu\text{g}/\text{ml}$ stock peptide in RPMI (peptide coated) (final concentration 10 $\mu\text{g}/\text{ml}$) was added to the cell pellet. One hundred microlitres of ^{51}Cr was added to cell pellet and the cells were incubated at 37°C for 1 hr. The cells were then washed twice with 25 RPMI/10%FCS through a FCS underlayer and resuspended at 10⁵/ml for target cells in the CTL assay.

(ii) Vaccinia (Vacc.) infected targets (B'-D', F'-J'): Vaccinia used for virus infected targets were the Murine 30 Polytope (Vacc Mu PT), with the Human Polytope (Vacc Hu PT) as the negative control. Cell lines infected by vaccinia were P815 (B'-D'), L929 (F') and EL-4 (G'-J'). The target cells were centrifuged at 1000rpm/5 min. The supernatant was discarded to approximately 200 μl and the 35 cells (approx. 10⁶ cells) infected with vaccinia at a multiplicity of infection (MOI) of 10:1 by adding 20 μl

vaccinia (10^9 pfu/ml) followed by incubation for 1 hr at 37°C. Five millilitres of RPMI/10%FCS was then added, cells mixed and incubated overnight at 37°C. These cells were subsequently centrifuged and supernatant discarded

5 into camdyne. One hundred microlitres of ^{51}Cr was added to cell pellet and the cells incubated at 37°C for 1 hr. The cells were then washed twice with RPMI/10%FCS through a FCS underlayer and resuspended at $10^5/\text{ml}$ for target cells in the CTL assay.

10

(iii) Influenza infected targets (A', E'): The A/PR/8/34 strain of Influenza virus was used for the Balb/c targets (A') and the reassortant A/Taiwan/1/86 (IVR-40) for the CBA targets (E'). Allantoic fluid was used as the negative control. Cell lines infected by influenza were P815 (A') and L929 (E'). Target cells were centrifuged at 1000rpm/5 min. and supernatant was discarded. Five hundred microlitres: 50 μl Influenza virus ($10^8/\text{ml}$ EID) or Allantoic Fluid, 50 μl ^{51}Cr , 400 μl RPMI/1%FCS was added to the cell pellet and incubated for 1 hr at 37°C. Ten millilitres of RPMI/10%FCS was added, mixed and incubated a further 2 hr at 37°C. The cells were then washed twice with RPMI/10%FCS through a FCS underlayer and resuspended at $10^5/\text{ml}$ for target cells in CTL assay.

25

(iv) Ovalbumin expressing targets (I'): EG7 cells are EL-4 cells transfected with an expression plasmid containing chicken ovalbumin cDNA (Moore MW, Carbone FR and Bevan BJ (1988) Introduction of soluble protein into

30 Class 1 pathway of antigen processing and presentation. Cell 54: 777-785.). These cells were maintained in RPMI/10% FCS. 2mM Hepes, 2mM Glutamine, 1mM Na Pyruvate, 100IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ Streptomycin. The plasmid was selected and maintained in Geneticin (G-418)

35 at 500 $\mu\text{g}/\text{ml}$ once per month. EL-4 cells with no peptide

(EL4 no pep) were used as the negative control. The cells were centrifuged at 1000rpm/5 min. and supernatant discarded to approximately 200 μ l. One hundred microlitres of ^{51}Cr was added to cell pellet and the cells incubated 5 at 37°C for 1 hr. The cells were then washed twice with RPMI/10%FCS through a FCS underlayer and resuspended at 10⁵/ml for target cells in the CTL assay.

3. CTL Assay. The restimulated splenocytes (5x10⁶/ml) 10 were dispensed (100 μ l) in triplicate at three Effector: Target ratios (50, 10, 2x10⁴ effector cells: 1x10⁴ target cells for the CTL assay. One hundred microlitres of target cells (10⁵/ml) were added to all effectors and control wells (Spontaneous release = 100 μ l media; Maximal 15 release = 100 μ l 0.5% SDS/ RPMI/10%FCS). Microtitre plates were centrifuged at 500rpm for 5 min. and incubated at 37°C for 6hr. Plates were re-centrifuged at 500rpm/5 min. and 25 μ l of supernatant was counted for ^{51}Cr release. Percentage Specific Lysis represents averages of 20 triplicate counts: 100 x (Test cpm - Spontaneous cpm)/(Maximal cpm - Spontaneous cpm).

The results are shown in Figure 9.

DNA vaccination experiment

25 The initial DNA vaccination experiments illustrate that the polytope can be delivered using DNA vaccination. In addition, that vaccination may be improved by the co-delivery of a cytokine gene (GM-CSF), although in this experiment the improvement is not statistically 30 significant.

The current system is clearly sub-optimal. Likely improvements would be the use of potentially better plasmid vectors e.g. the vectors from Vical, San Diego (Sedegah M, R Hedstrom, P Hobart, SL Hoffman, 1994. 35 Protection against malaria by immunisation with plasmid

DNA encoding circumsporozoite protein. PNAS 91, 9866-9870) and the use of better delivery systems (to IM injection) employing a gene gun (Sun WH., Burkholder JK., Sun J., Culp J., Lu XG., Pugh TD., Ershler WB, Yang NS. IN VIVO
5 CYTOKINE GENE TRANSFER BY GENE GUN REDUCES TUMOUR GROWTH IN MICE. Proceedings of the National Academy of Sciences of the United States of America. 92:2889-2893, 1995.). In addition priming against CTL epitopes usually requires CD4 T cell help¹⁷ thus the inclusion helper epitopes or
10 proteins in the construct may improve the level and reliability of CTL priming by the murine DNA vaccine polytope.

Lack of "Original antigenic sin" or the ability of a
15 polytope to raise immune responses to all the epitopes in a polytope when the individual has already got a response to one of the epitopes.

Introduction

20 Original antigenic sin is a term given to an antibody based phenomena whereby an existing antibody response to an epitope prevents the raising of an immune response to a second epitope when that epitope is present on the same protein as the first epitope (Benjamini E.,
25 Andria M.L., Estin C.D., Notron, F.L. & Leung C.Y. (1988) Studies on the clonality of the response to an epitope of a protein antigen. Randomness of activation of epitope - recognizing clones and the development of clonal dominance. *J. Immunol.* 141,55.). The reason for this
30 phenomena is that large population of primed B cells specific for the first epitope bind and mop up all the available antigen before a naive B cell specific for the second antibody has a chance to bind the antigen, process it and present it to T helper cells. A similar situation
35 might occur when an individual is vaccinated with a polytope when he/she already has a response to one of the

epitopes in the polytope. The existing CTL might kill all the polytope expressing cells before any of the other epitopes can be presented to naive T cells.

Method

5 To test this possibility mice (Balb/c) were infected with 10^4 pfu of Murine cytomegalovirus (MCMV) (K181 strain - Scalzo et al. 1995) and left for 5 weeks at which point strong CTL responses specific for the MCMV epitope, YPHFMPTNL, had developed (Scalzo et al. 1995 - Fig 2A).
10 These mice were then given the murine polytope vaccinia and spleen cells assayed 10 days later for CTL specific for the three other epitopes presented by the polytope in this strain of mouse (RPQASGVYM, Lymphocytic choriomeningitis virus nuclear protein, H-2L^d; TYQRTRALV,
15 influenza nuclear protein, H-2K^d and SYIPSAEKI, P. Berghei circumsporozoite protein, H-2K^d).

Results

Responses to each of the three new epitopes was observed following polytope vaccination, illustrating that
20 the YPHFMPTNL specific CTL did not prevent priming of CTL specific for RPQASGVYM, TYQRTRALV and SYIPSAEKI when all four epitopes are presented together in the polytope. (Control animals receiving the human polytope vaccinia instead of the murine polytope vaccinia, showed only
25 YPHFMPTNL specific CTL).

This series of experiments illustrate that if a polytope was, for instance, designed to cover a variety of different diseases, an individual receiving such polytope vaccine, but who had already been exposed to one of the
30 target diseases would still be immunised against the remaining CTL epitopes in the polytope.

As will be apparent to those skilled in the art the present inventors have shown that the natural flanking sequences of CTL epitopes are not required for class I processing, that is each epitope within the polyepitope protein was always efficiently processed and presented to

appropriate CTL clones by autologous polyepitope vaccinia infected target cells. It will be apparent to those skilled in the art that the polytopes may include sequences not naturally found to flank the epitopes.

5 As discussed above the present invention can be used with a range of epitopes. A range of epitopes are now available on an Internet address which is described in Brusic *et al* Nucleic Acids Research, 1994, 22; 3663-5.

10 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as
15 illustrative and not restrictive.

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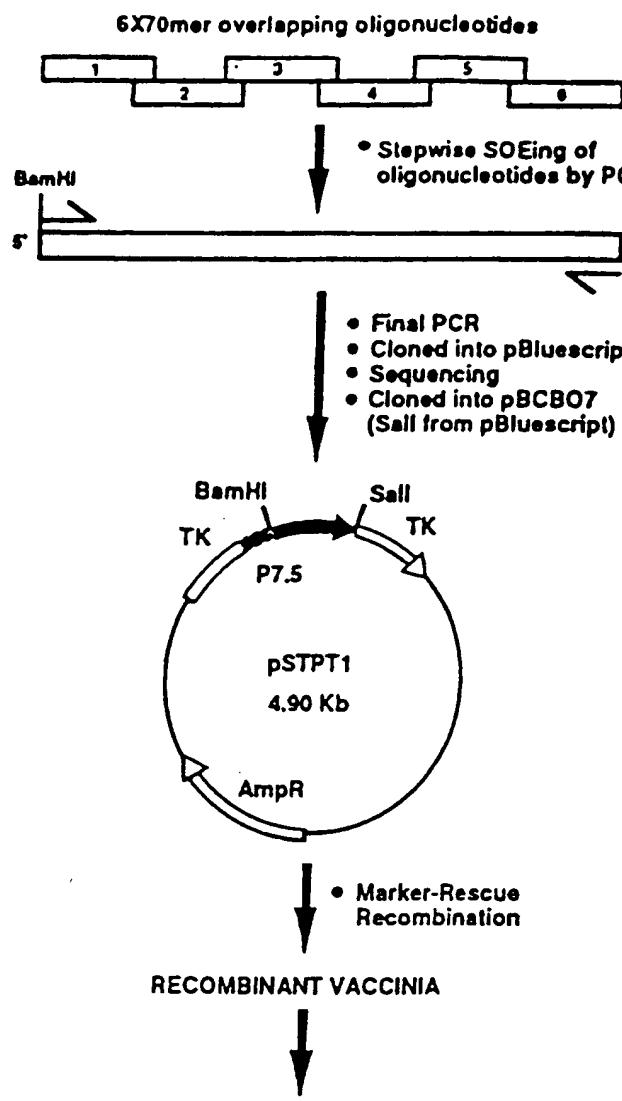
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CLAIMS:-

1. A recombinant polyepitope cytotoxic T lymphocyte vaccine, the vaccine comprising at least one recombinant protein including a plurality of cytotoxic T lymphocyte epitopes from one or more pathogens, wherein the at least one recombinant protein is substantially free of sequences naturally found to flank the cytotoxic T lymphocyte epitopes.
2. A recombinant polyepitope cytotoxic T lymphocyte vaccine as claimed in claim 1 in which the at least one recombinant protein does not include sequences naturally found to flank the cytotoxic T lymphocyte epitopes.
3. A recombinant polyepitope cytotoxic T lymphocyte vaccine as claimed in claim 1 or claim 2 in which recombinant protein includes at least three cytotoxic T lymphocyte epitopes.
4. A recombinant polyepitope cytotoxic T lymphocyte vaccine as claimed in any one of claims 1 to 3 in which the cytotoxic T lymphocyte epitopes are from multiple pathogens.
5. A polynucleotide including at least one sequence encoding a plurality of cytotoxic T lymphocyte epitopes from one or more pathogens, wherein the at least one sequence is substantially free of sequences encoding peptide sequences naturally found to flank the cytotoxic T lymphocyte epitopes.
6. A polynucleotide as claimed in claim 5 in which the at least one sequence is free of sequences encoding peptide sequences naturally found to flank the cytotoxic T lymphocyte epitopes.
7. A polynucleotide as claimed in claim 5 or claim 6 in which the at least one sequence encodes at least three cytotoxic T lymphocyte epitopes.
8. A polynucleotide as claimed in any one of claims 5 to 7 in which the at least one sequence encodes cytotoxic T lymphocyte epitopes are from multiple pathogens.

9. A nucleic acid vaccine, the vaccine comprising the polynucleotide as claimed in any one of claims 5 to 8 and an acceptable carrier.
10. A vector including the polynucleotide as claimed in 5 any one of claims 5 to 8.
11. A vector as claimed in claim 10 in which the vector is selected from the group consisting of vaccinia vectors, avipox virus vectors, bacterial vectors, virus-like particles (VLP's) and rhabdovirus vectors.
- 10 12. A vaccine formulation, the vaccine formulation comprising the recombinant protein as claimed in any one of claims 1 to 4 and an acceptable carrier and/or adjuvant.
13. A vaccine formulation as claimed in claim 12 in 15 which the formulation includes ISCOMs.



M**S**TNSFLRGRAYGL QAKWRLQTL-
 EENLLDFVRF SVRDRRLARLKEHVIQNAF-
 YPLHEQHGMHLAAQGMAY DTPLIPLTIF-
 IVTDFSVIK **NNLVSGPEH**

Polytope Protein Sequence

FIGURE 1

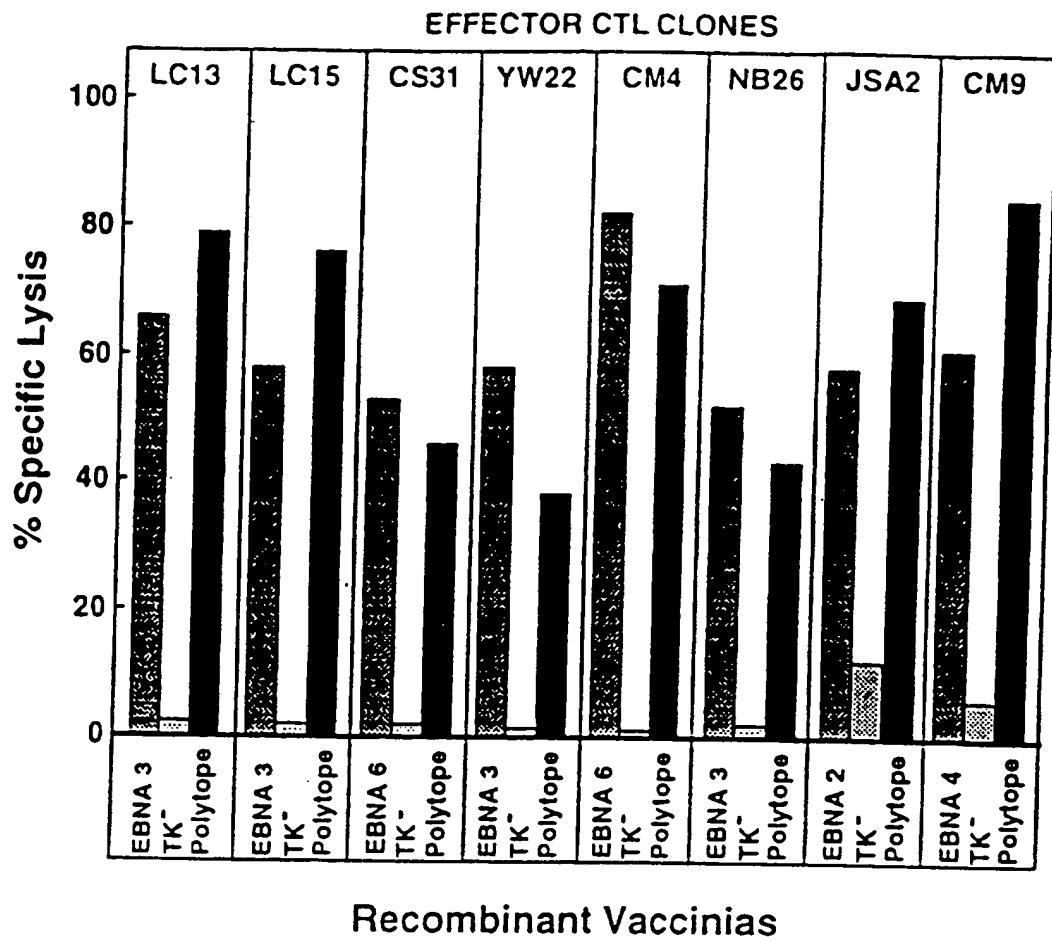


FIGURE 2

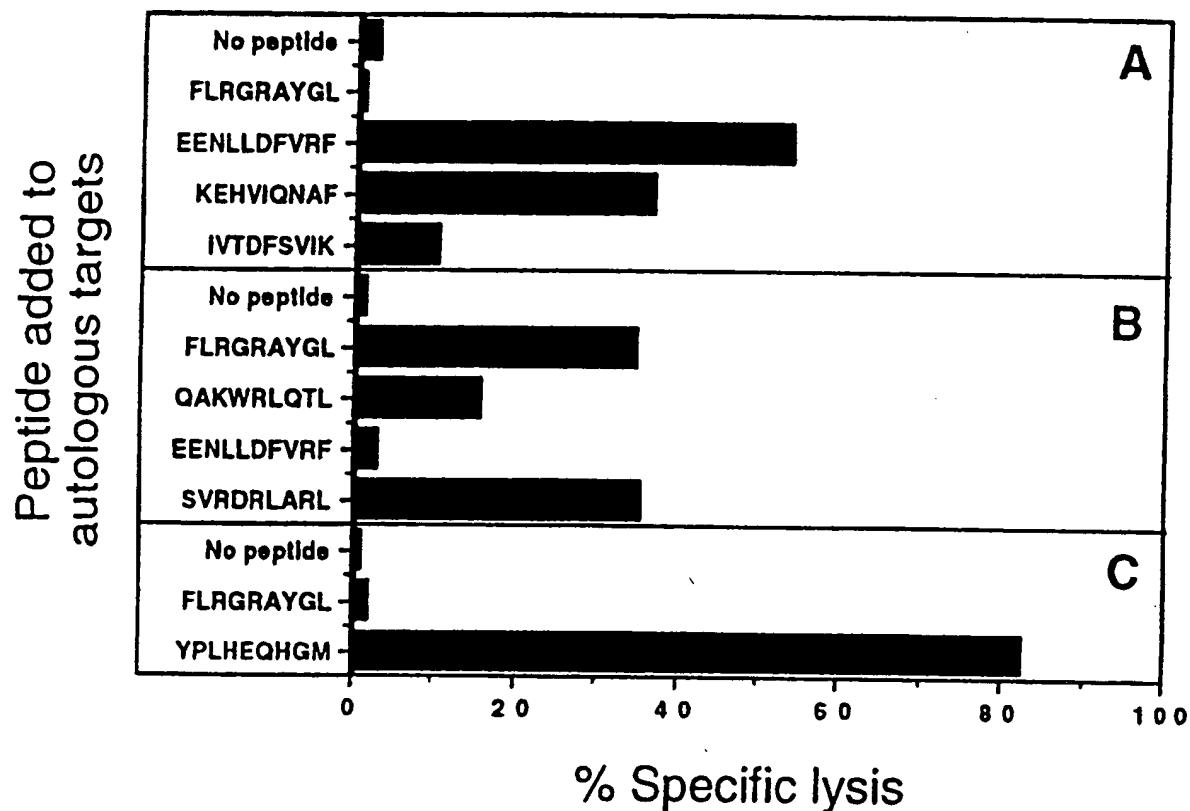


FIGURE 3

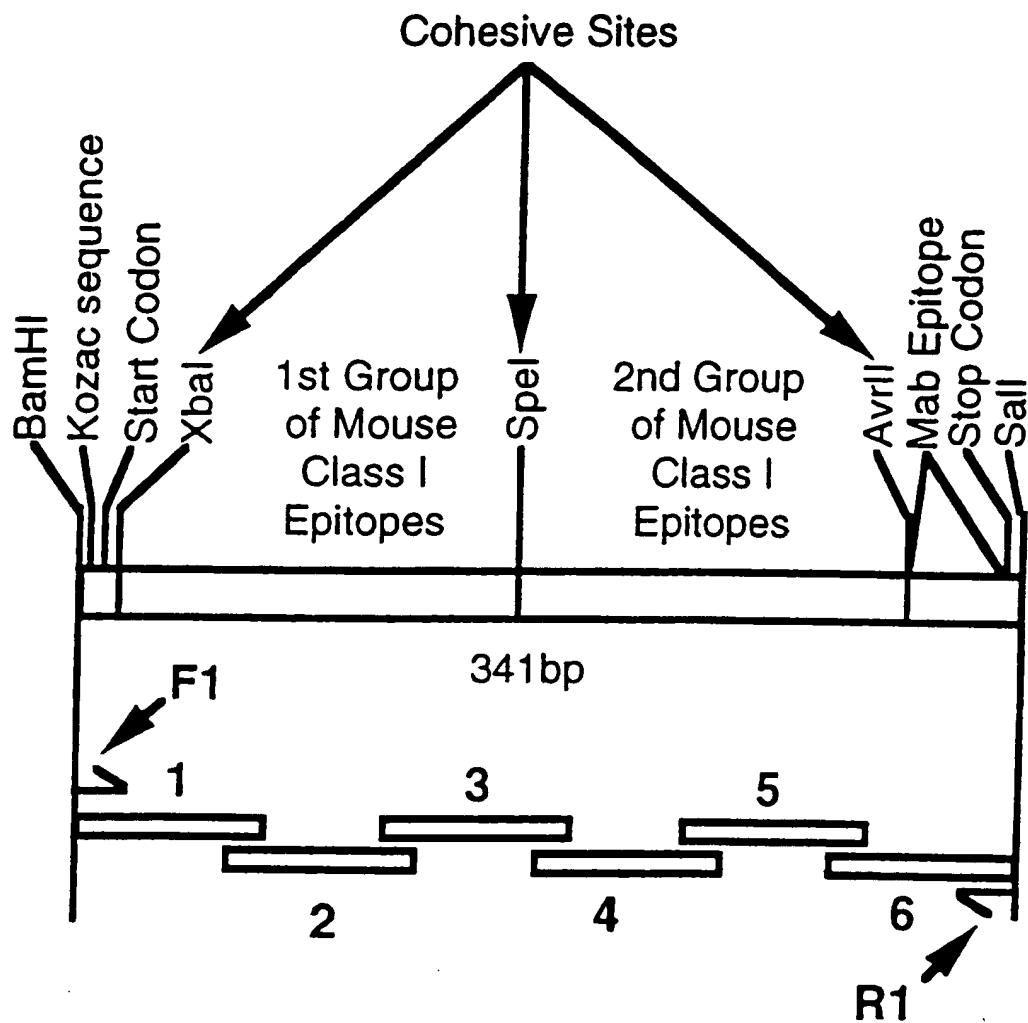


FIGURE 4

BamHI Kozac
GGATCC CCACC

Start XbaI
M S R A S N E N M D A M S I I N F E K L T Y Q R T R A L V
ATG TCTAGA GCC AGC AAC GAG AAC ATG GAC GCC ATG
AGC ATC ATC AAC TTC GAG AAC CTT

SpeI
S D Y E G R L I Y P H F M P T N L T S S G P S N T P P E I
TAC CCA CAC TCA ATG CCT AAC M C C T G
AGC GAC TAC GAG GGC AGG CTC ATC

AvII
F A P G N Y P A L S Y I P S A E K I E E G A I V G E I
TTC GCC CCA GGC AAC TAC CCT GCG
AGC TAC ATC CCA AGC GCC GAG AAC ATC

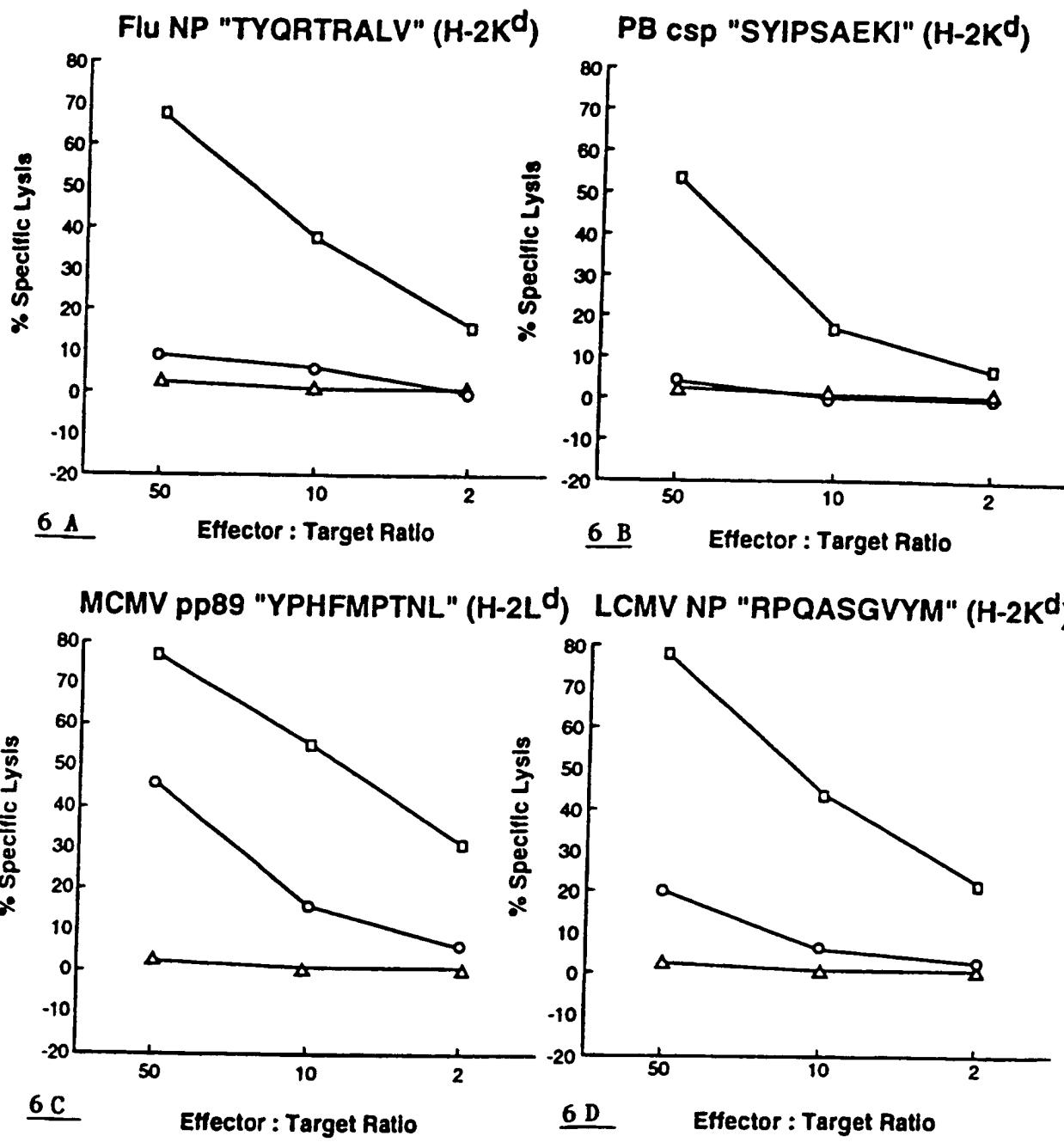
Mab epitope
R P Q A S G V Y M P R N N L V S G P E H L Z
AGG CCT CAG GGC AGC GGC AAC TAC
CCC AGG AAC GGC GGC AAC TAC

Stop Sall
T A G G T C G A C

FIGURE 5

Balb/c

6/15



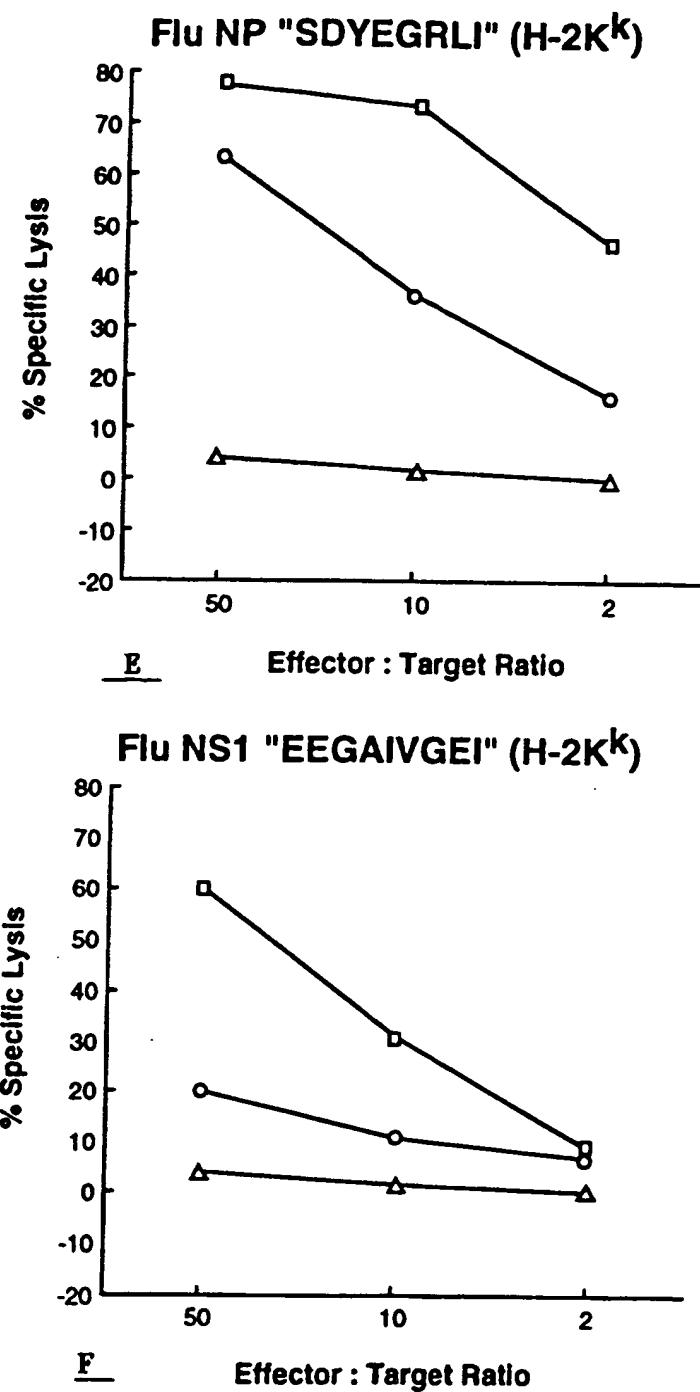
LEGEND

- Bulk Splenocytes from Polytope Vaccinated Mice
- ▲ No peptide Control
- Bulk Splenocytes from TK- Vaccinated Mice

FIGURE 6

CBA

7/15



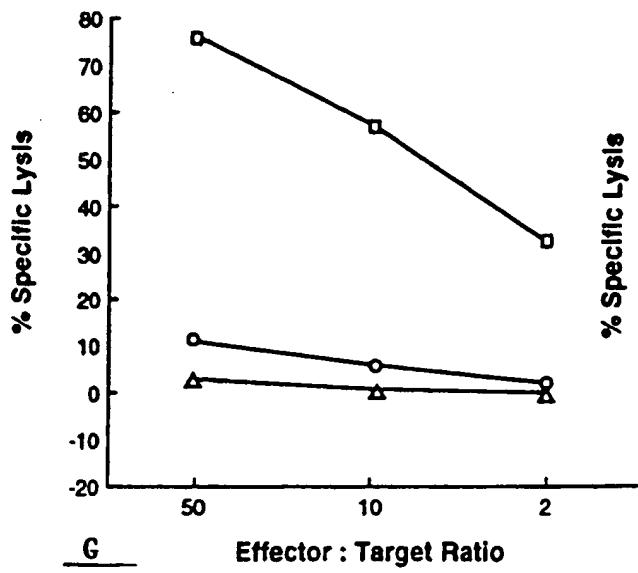
LEGEND

- Bulk Splenocytes from Polytope Vaccinated Mice
- △— No peptide Control
- Bulk Splenocytes from TK- Vaccinated Mice

FIGURE 6

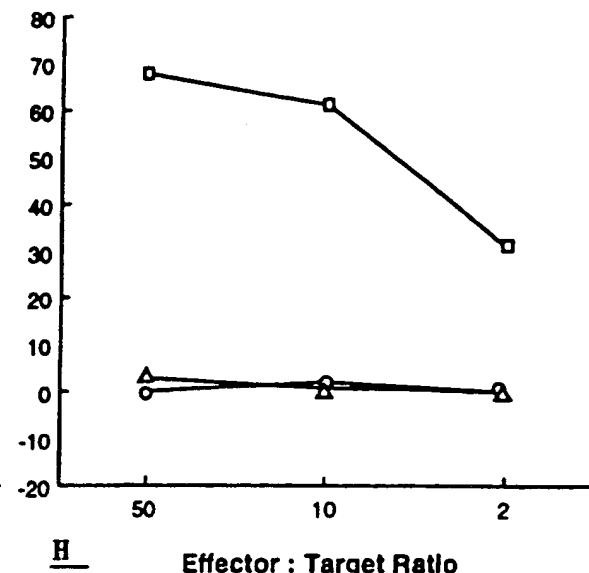
C57BL/6

Flu NP "ASNENMDAM" (H-2D^b)

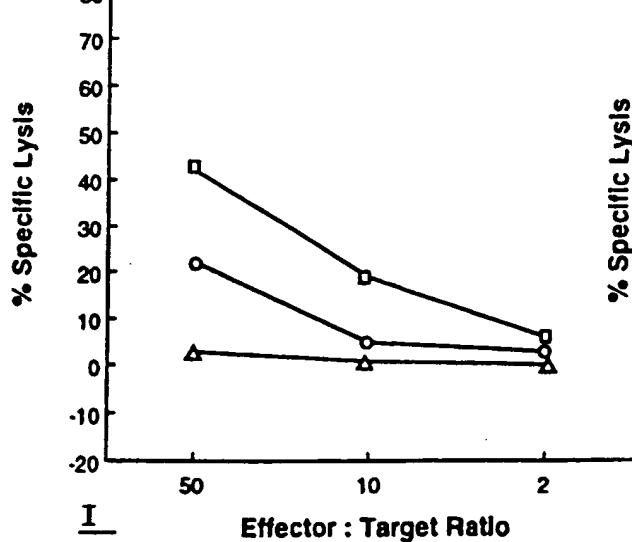


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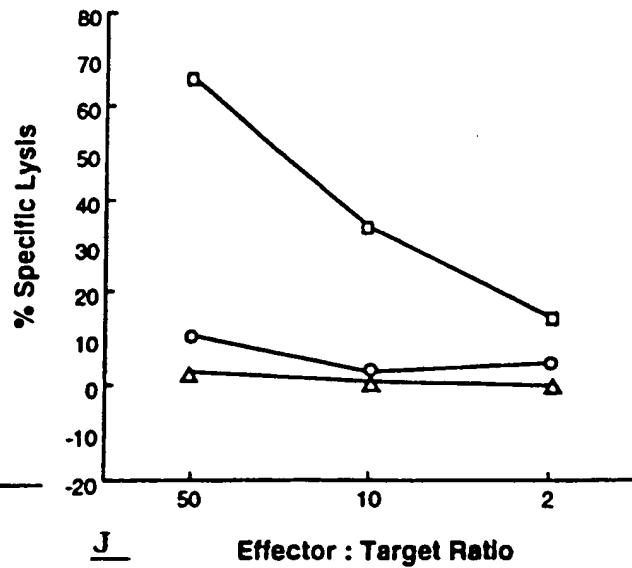
Ad5 E1A "SGPSNTPPEI" (H-2D^b)



Ovalbumin "SIINFEKL" (H-2K^b)



Sendi NP "FAPGNYPAL" (H-2K^b)



LEGEND

- Bulk Splenocytes from Polytope Vaccinated Mice
- △ No peptide Control
- Bulk Splenocytes from TK- Vaccinated Mice

FIGURE 6

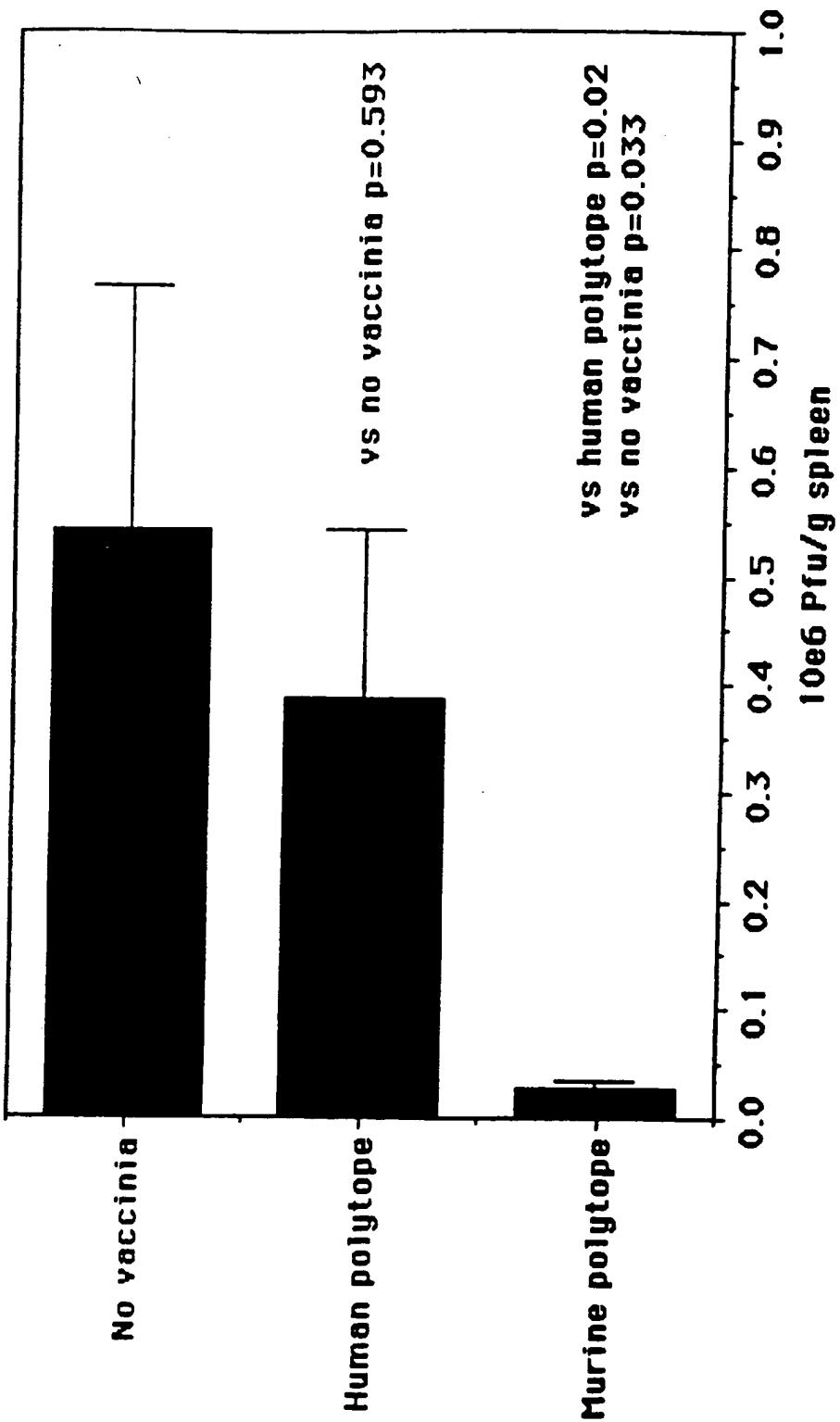


FIGURE 7

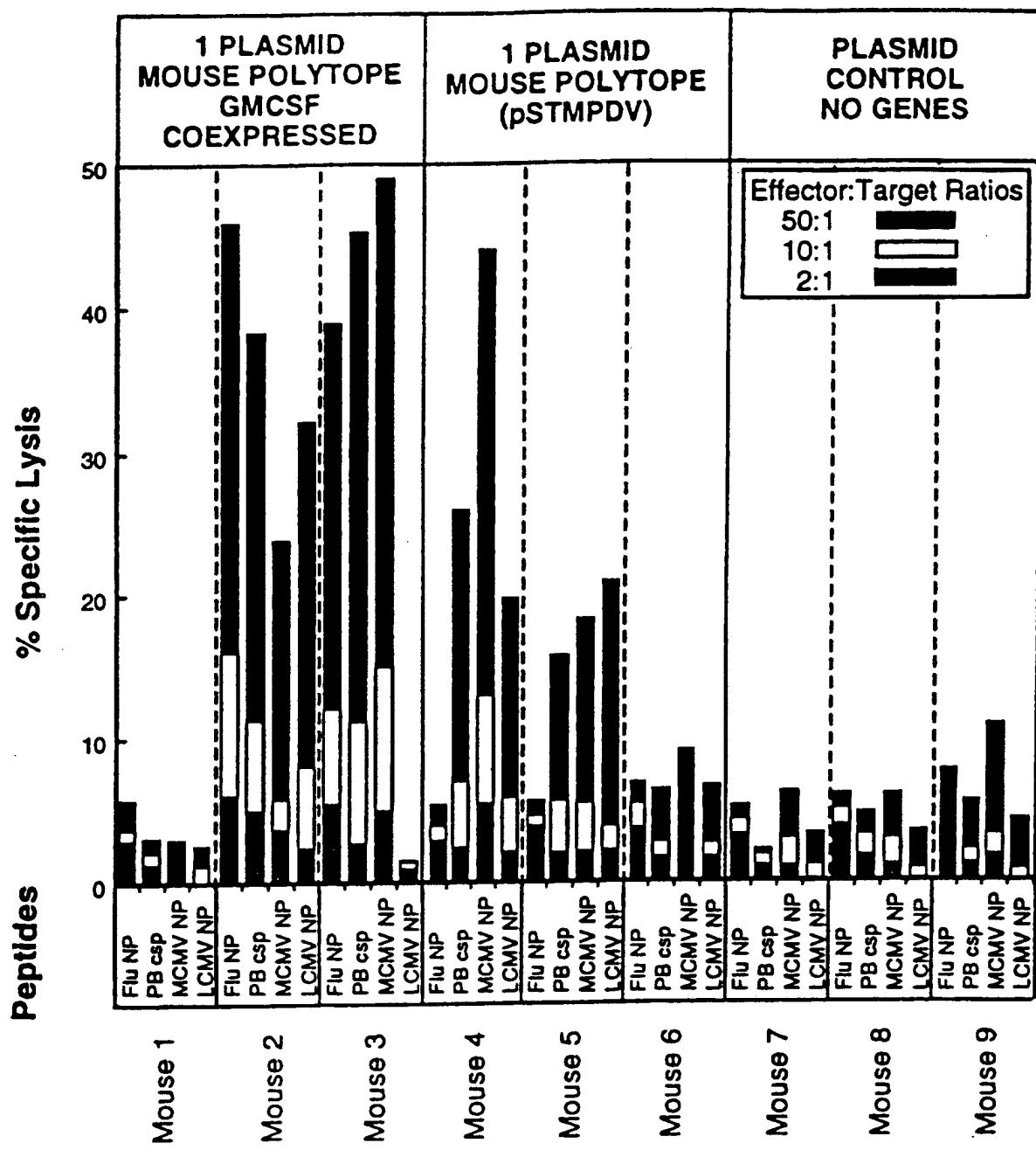


FIGURE 8

Balb/c I.P. Vaccinated Mice

PEPTIDE COATED TARGETS

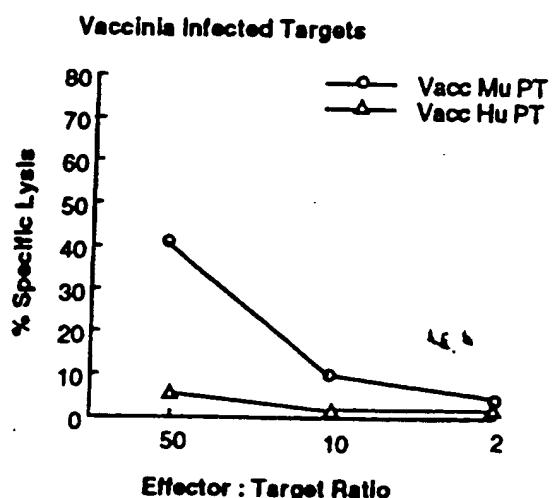
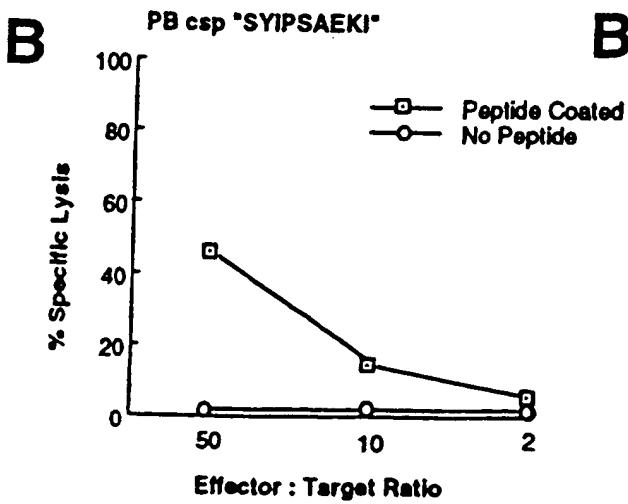
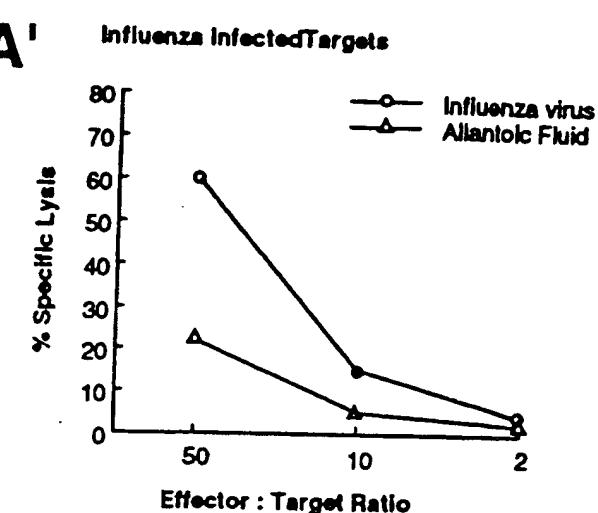
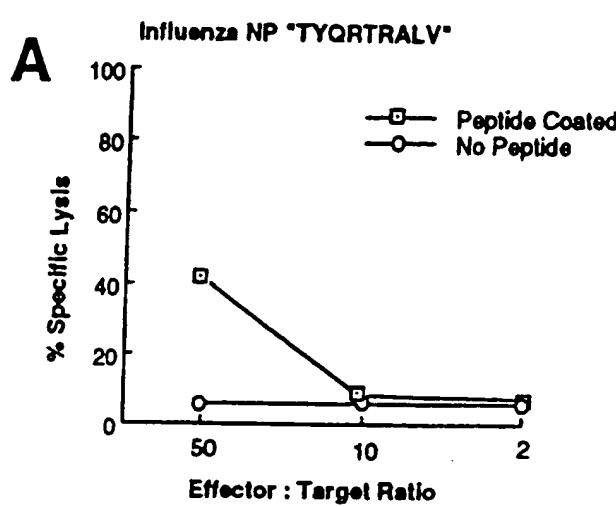


FIGURE 9

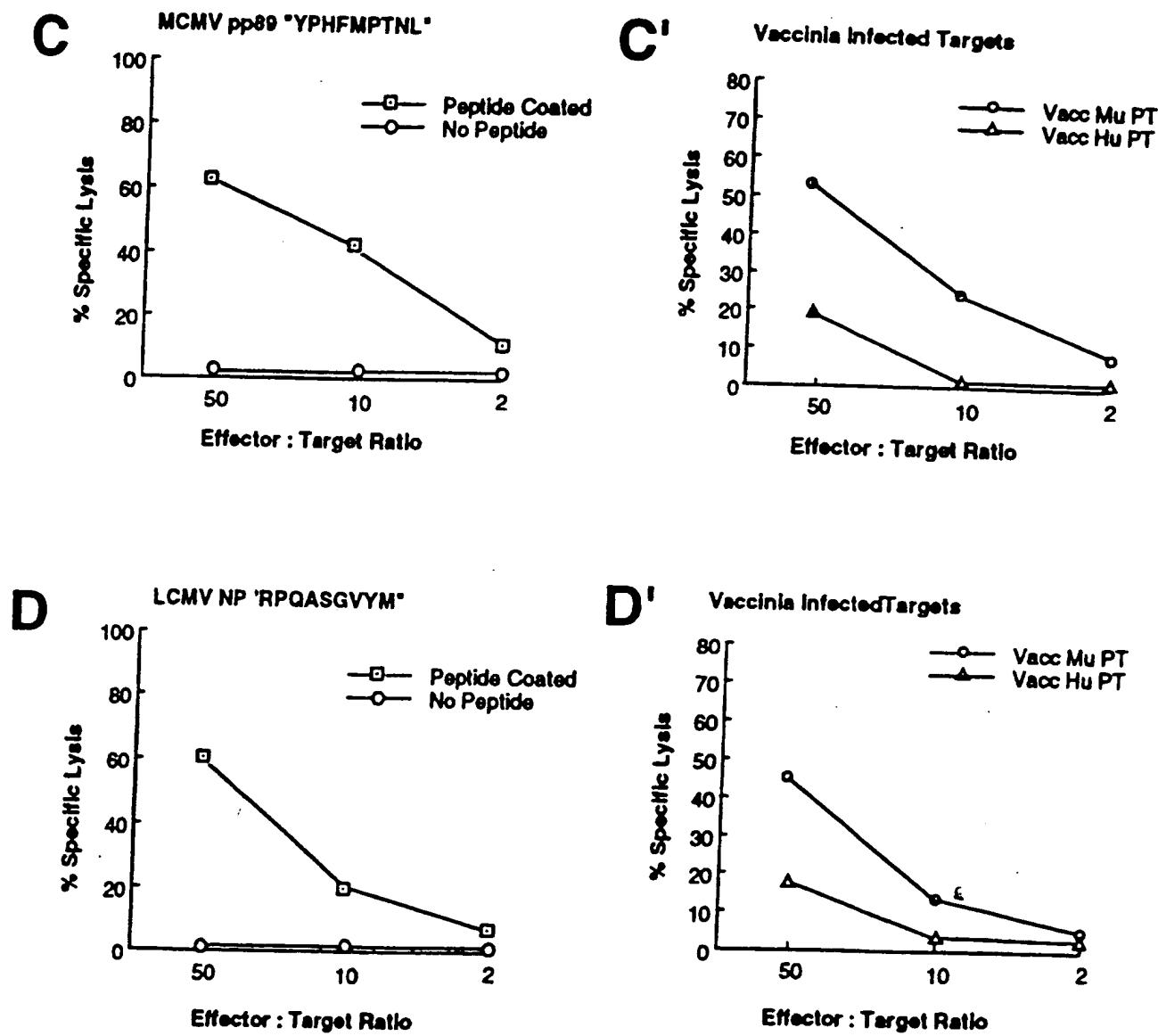
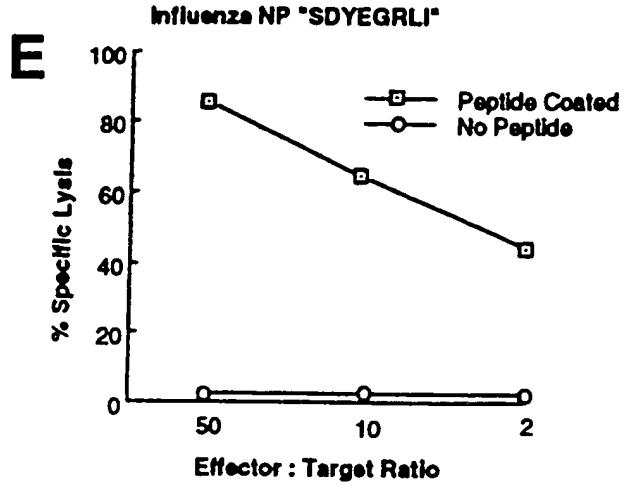


FIGURE 9

CBA I.P. Vaccinated Mice

PEPTIDE COATED TARGETS



VIRUS INFECTED TARGETS

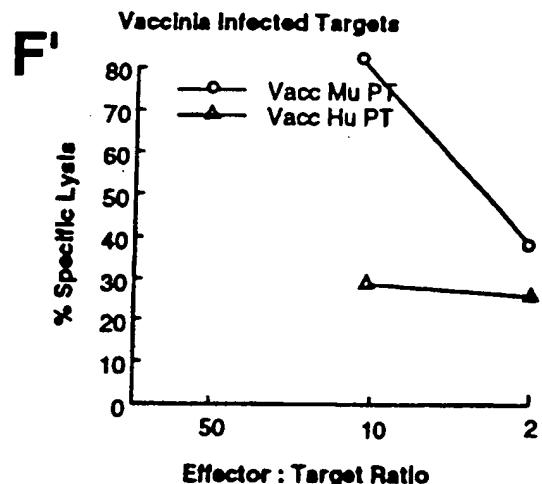
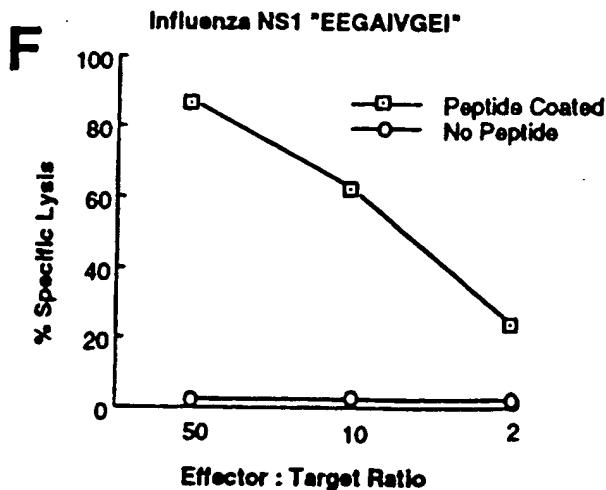
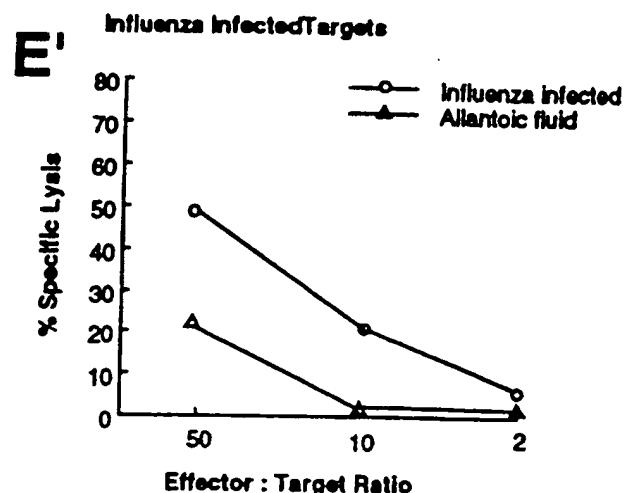
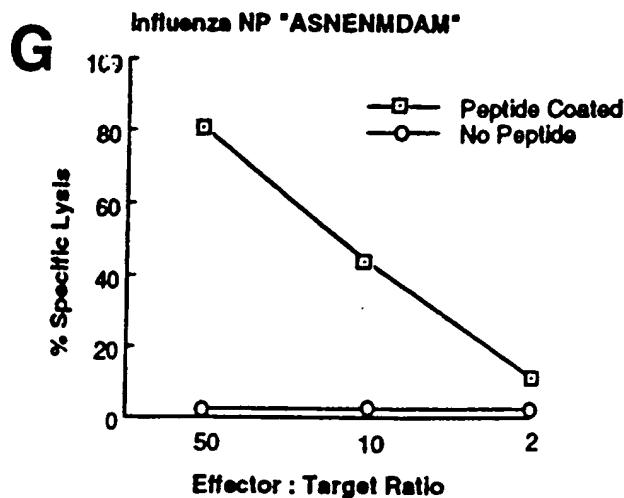


FIGURE 9

C57BL/6 I.P. Vaccinated Mice

PEPTIDE COATED TARGETS



TUMOUR AND VIRUS INFECTED TARGETS

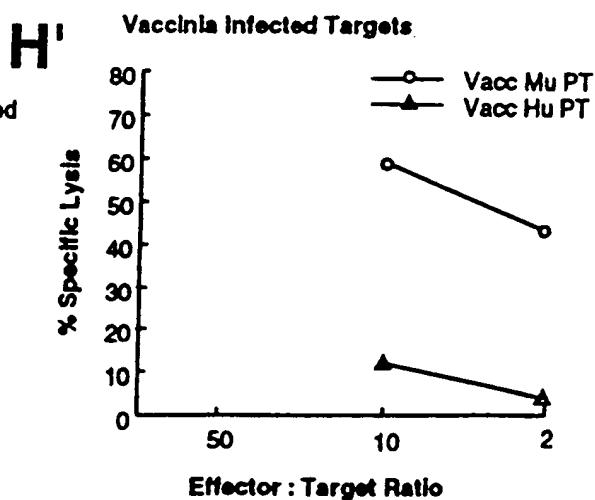
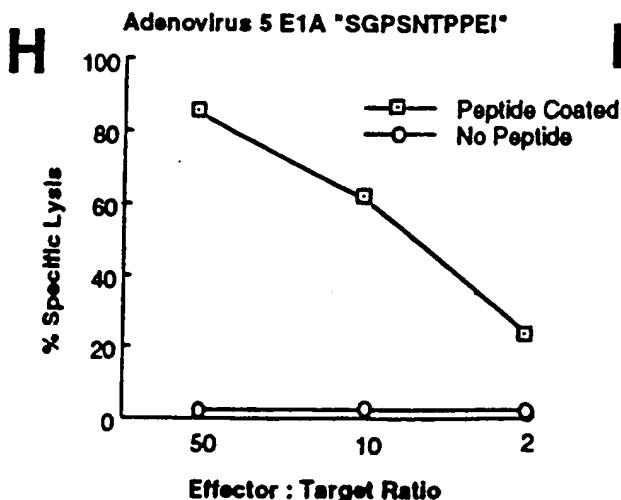
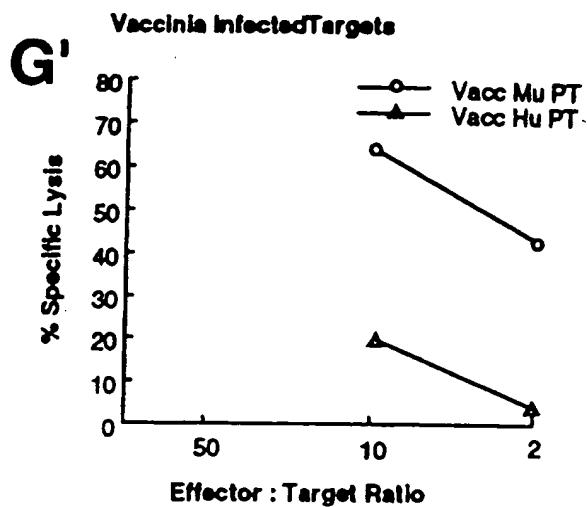


FIGURE 9

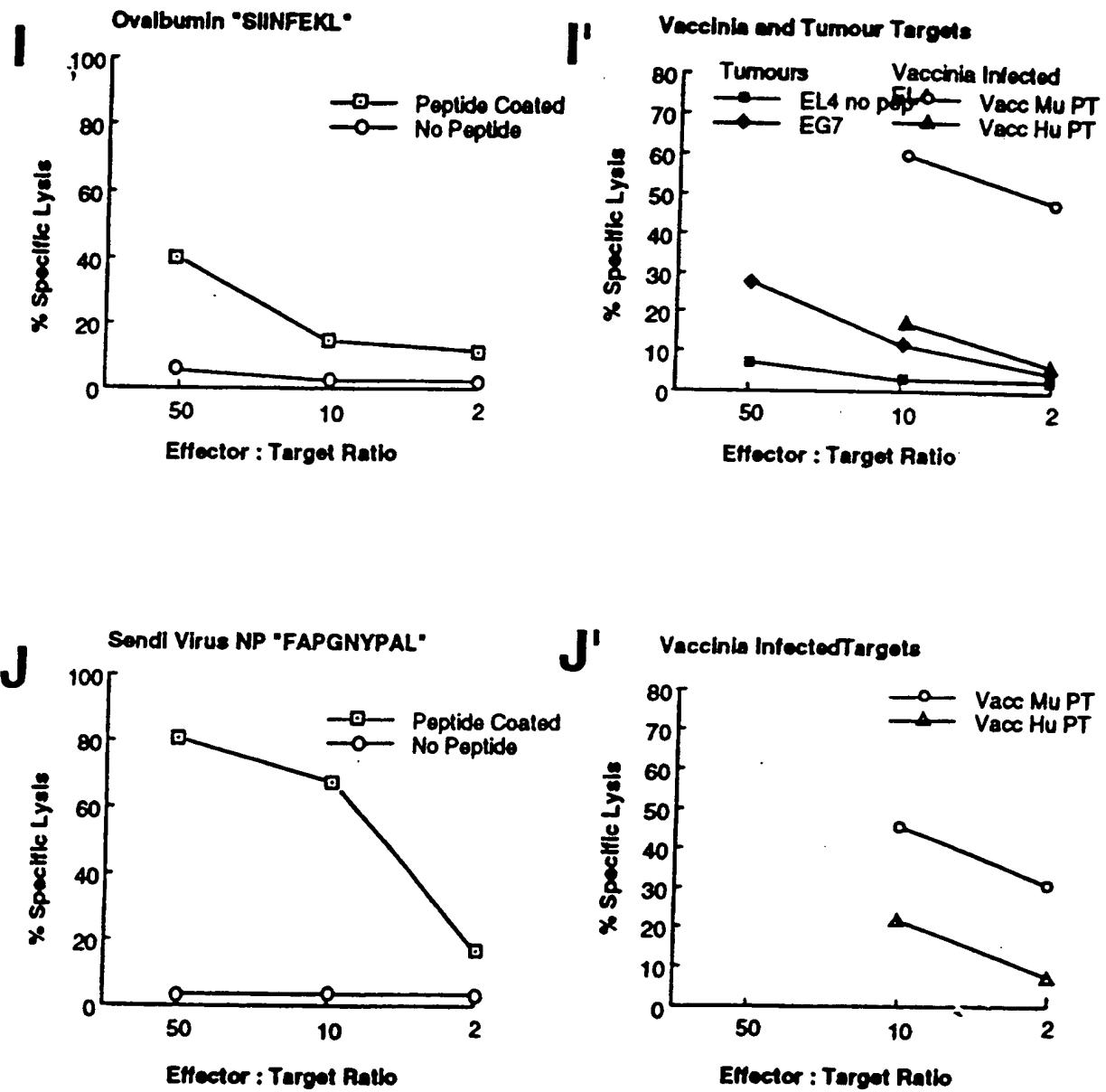


FIGURE 9

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl⁶: A61K 39/00 39/395 C12N 15/86 15/12 15/13 C07K 19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 39/- C12N 15/-

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DERWENT: WPAT, CASM, KEYWORDS: CYTOTOXIC T CELL^c, CTL, POLYVALEN[;], POLYEPITOP[;], MULTIEPITOPmULTIEPITOP[;], MULTIPLE EPITOP[;], VACC[;], IMMUNOGEN[;], ANTIGEN[;]

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ^a	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Virology, Volume 67(1), 1993, pages 348-352, Whittington et al. "A string-of-beads vaccine, comprising linked minigenes, confers protection from lethal-dose virus challenge", see abstract, results.	1-13
X	WO 93/25575 (United States of America) 23 December 1993, see page 5 lines 3 to 17, examples, claim 2.	1-13
PX	WO 94/26785 (United States of America) 24 November 1994, see examples, claims.	1-13



Further documents are listed in the continuation of Box C



See patent family annex

°	Special categories of cited documents:	
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

29 September 1995

Date of mailing of the international search report

13 OCTOBER 1995

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C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94/18234 (United Biomedical Inc.) 18 August 1994, see abstract, claims, figure 1.	1-3, 5-7
Y		4, 8-13
Y	Journal of Immunology, Volume 149(1), 1992, pages 269-276, Rogers et al, "Augmentation of in vivo cytotoxic T lymphocyte activity and reduction of tumor growth by large multivalent immunogen", see abstract.	1-13
Y	WO 93/22343 (The Rockefeller University) 11 November 1993, see pages 1 to 5, claims, figures 7 and 8.	1-13
A	WO 90/11085 (Medical Biology Institute) 4 October 1990, whole document	1-13

Information on patent family members

PCT/AU 95/00461

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report			Patent Family Member		
WO	9325575	AU	45300/93	EP	644894
WO	9426785	AU	70173/94		
WO	9011085	EP	422164	US	5045320
WO	9418234	AU	61740		

END OF ANNEX

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